

JOHN I. GALTIN
RALPH SNYDERMAN

INFLAMMATION

*Basic Principles and
Clinical Correlates*

Third Edition

Associate Editors:

DOUGLAS T. HEARON BARTON F. HAYNES
CARL NATHAN



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CHAPTER 6

Platelets: Their Role in Hemostasis, Thrombosis, and Inflammation

Aaron J. Marcus

PLATELET MORPHOLOGY AND PLATELET PRODUCTION

Platelets are the keystone of the hemostatic arch. In contrast to the thrombocytes of birds (1) and reptiles (2), mammalian platelets are cytoplasmic fragments, devoid of nuclei, and are derived from megakaryocytes in the marrow. Megakaryocytes themselves originate from pluripotent stem cells of hematopoiesis (3). *In vitro* culture studies have shown that at least two stages of megakaryocyte progenitors can be demonstrated: the burst-forming unit-megakaryocyte and the colony-forming unit-megakaryocyte. Developmental stages of these progenitors are controlled by various cytokines. As the colony-forming unit-megakaryocyte matures, these cells become recognizable as such. An endomitotic process then leads to multilobulation of megakaryocyte nuclei. Under normal circumstances, megakaryocyte ploidy can range from 8N to 32N. However, platelet production and consumption are the main determinants of ploidy number.

The megakaryocyte cytoplasm fragments along demarcation membranes which are formed by enfolding of cytoplasmic membrane material. The size of the demarcation zones is the ultimate determinant of platelet size. When there is a demand for increased platelet production, the demarcation zones become larger. This is reflected in measurements of mean platelet volume (MPV), which is larger in thrombocytopenic states due to increased platelet consumption. The total mass of platelets in the

circulation is the main determinant of platelet count. This is observed in platelet diseases such as the Bernard-Soulier syndrome and the May-Hegglin anomaly, wherein thrombocytopenia is accompanied by an increase in MPV (4). One third of circulating platelets are in the spleen (5).

It is possible to increase platelet production therapeutically through administration of recombinant human thrombopoietin or megakaryocyte growth and development factor. This material elevates the platelet count in a dose-dependent manner within 4 to 6 days after initiation of treatment (6).

Although platelets look somewhat uncomplicated on microscopic examination, this appearance is deceptive. The platelet is a functional entity, as evidenced by the critical roles it plays in hemostasis, coagulation, thrombosis, and as a participant in the inflammatory response. Platelets in the circulation appear to be passive, smooth discs, but on their surface they possess mechanisms for recognizing a site of injury that can trigger subsequent adhesion, spreading, activation, and release (5,7,8).

The discoid, circulating, "resting" platelet contains a circumferential band of microtubules that serves to maintain its discoid shape. The platelet membrane, which is the only cell plasma membrane derived from cytoplasmic components, contains invaginations. These form the surface-connected open canalicular system. The area beneath the plasma membrane, known as the sol-gel zone, contains abundant quantities of actin and myosin. In all likelihood, these proteins are critical for platelet shape change and spicule formation, which is part of the response to agonists inducing platelet activation (5). The platelet counterpart of endoplasmic reticulum is the dense tubular system, which is a reservoir for calcium storage. Other major platelet cytoplasmic components include mitochondria, peroxisomes, platelet α granules,

A. J. Marcus: Division of Hematology and Medical Oncology, Department of Medicine, Veterans Affairs Medical Center, New York, New York 10010.

This chapter is dedicated to the memory of Dr. Ira M. Goldstein.

and dense granules. Platelets also have abundant storage pools of glycogen.

A broad spectrum of proteins has been identified in platelet granules. It is controversial whether intracellular platelet proteins were adsorbed from plasma or were actively synthesized in the megakaryocyte. Probably both mechanisms play a role. For example, platelet albumin, fibrinogen, and immunoglobulin G (IgG) quantities seem to be proportionate to their plasma concentrations. Platelet factor-4 (the antiheparin protein), β -thromboglobulin, and von Willebrand's factor (vWF) do originate in the megakaryocyte. The platelet dense granule compartment contains adenosine diphosphate (ADP), adenosine triphosphate (ATP), serotonin, and calcium. ADP is the most important platelet agonist and recruiting agent in human plasma.

Platelets circulate for approximately 7 to 10 days. Normal platelet counts range from 150,000 to 440,000 per microliter. Normal platelets have a diameter of $3.6 \pm 0.7 \mu\text{m}$. The platelet thickness is $0.9 \pm 0.3 \mu\text{m}$, and the volume of the average platelet is $7.06 \pm 4.85 \text{ fL}$. When thrombopoiesis is increased, the MPV increases accordingly. This is a reflection of an increase in size of the megakaryocytes. It is generally accepted that MPV decreases as platelets age. Platelet α granules are the major determinants of platelet density, which varies about 5% among different subjects. Figure 6-1 depicts the major aspects of platelet morphology.

Despite major advances in comprehension of platelet biochemistry and function, one of the most important properties of platelets—maintenance of vascular integrity—is not understood. Yet, it forms the main basis for therapy with platelet transfusions. Acute thrombocytopenia usually is associated with spontaneous cutaneous and mucous membrane hemorrhage. It has been assumed that the hemorrhage is caused by loss of structural and functional integrity at the level of the vascular endothelium. This breakdown leads to increases in capillary fragility and permeability. The platelet components responsible for maintenance of vascular integrity have not been identified, and there is no explanation for their hypothetical mechanism of action. Only viable, intact platelets can halt thrombocytopenic bleeding, which suggests that the platelet "vascular integrity factor" is a metabolic entity.

Platelets also mediate clot retraction—a function that can be observed *in vitro*. Clot retraction requires ATP, glucose, and a highly consolidated coagulum as promoted by thrombin. The process of clot retraction may be mediated by an interaction between gpIIb/IIIa and the actin cytoskeleton. Cytoskeletal proteins such as talin and vinculin also appear to be involved in clot retraction. As anticipated, clot retraction is weak to absent in thrombasthenia, owing to a deficiency or absence of gpIIb/IIIa . Clot retraction is defective in thrombocytopenic states and during excessive fibrinolysis. In the latter condition, the retracted clot becomes liquefied *in vitro*.

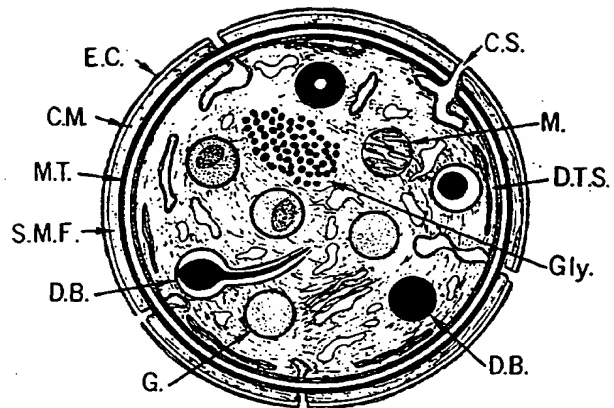


FIG. 6-1. Diagram of platelet ultrastructure in an equatorial plane. The peripheral zone, exterior to the plasma membrane, contains glycoprotein-rich material and is considered as glycocalyx (EC). The glycocalyx is the locale of platelet receptors for agonists and inhibitors. The glycocalyx is also the site of signal transduction. The platelet plasma membrane (CM) is a classic lipid bilayer with a unique phospholipoprotein component that can rearrange itself in response to agonist stimulation. This rearrangement confers coagulation-promoting properties on the activated platelet. The cell membrane and submembrane filament area (SMF) are the site of invaginations at specific sites. These invaginations comprise lining channels of the surface-connected open canalicular system (CA). The submembrane filaments are composed of actin from the membrane cytoskeleton.

In response to activation, a platelet surface membrane rearrangement occurs, forming the catalytic phospholipoprotein surface for assembly of activated coagulation proteins. This assembly does not take place on the surface of resting platelets. Also in response to activation, the platelet submembrane filaments form parallel structures that are the basis of filopodia or spicule formation—the "marker" for stimulated platelets.

The circumferential system of microtubules (MT) constitutes the entire cytoskeleton and it is proposed, although controversial, that the microtubule system is responsible for maintenance of the disk shape of unstimulated platelets. The platelet cytoplasm itself is also known as the sol-gel zone. It contains microfilaments, submembrane filaments, the circumferential band of microtubules, and glycogen. Other formed elements in the sol-gel zone include mitochondria (M), dense bodies (DB), and lysosomes.

Platelet granules (G) are of two types. One is lysosomal, and those of the other type contain adhesive-type proteins and agonists that are released into the external milieu upon platelet activation. These include fibrinogen, von Willebrand's factor, thrombospondin, fibronectin, and P-selectin; P-selectin translocates to the platelet surface on activation. Also present are platelet factor-4, β -thromboglobulin, and platelet-derived growth factor (PDGF). In addition, transforming growth factor- β , coagulation factor V, plasma activator inhibitor-1 (PAI-1), protein S, and high-molecular-weight kininogen. The major agonists are adenosine diphosphate (ADP) and serotonin. The dense tubular system (DTS) and surface-connected canalicular system are actually membrane systems within the platelet that are counterparts of the sarcoplasmic reticulum in other cells. (From White JG. Platelet granule disorders. *Crit Rev Oncol Hematol* 1996;4:337, copyright CRC Press, Inc., Boca Raton, Florida, with permission.)

MECHANISMS OF HEMOSTASIS

The hemostatic process represents a series of physiologic and biochemical reactions that culminate in the arrest of hemorrhage from blood vessels that have been severed or mechanically traumatized. Hemostasis is accomplished by the interaction of three systems: components of the vasculature itself, including endothelial cells; blood platelets; and plasma proteins of the intrinsic and extrinsic coagulation systems (9,10). Qualitative or quantitative deficiencies in one of these systems can result in defective hemostasis or coagulation, or both, leading to a mild, moderate, or severe hemorrhagic diathesis (5,7).

The efficiency of the hemostatic process leads to a paradox: at sites of pathologic damage, such as a necrotic or fissured atherosclerotic plaque, these structures serve as agonists for unwanted activation of hemostasis and promotion of blood coagulation. This culminates in arterial or venous thrombosis at critical sites such as the coronary or cerebral circulation. Thrombosis therefore can be classified as a misdirected form of hemostasis. It is the major complication of atherosclerotic disease (8) and accounts for 50% of mortality in the United States, Europe, and Japan (11-13). Last year in the United States alone, there were 2,500,000 thrombotic episodes, leading to 600,000 deaths.

Primary Hemostasis

Interruption of blood vessel continuity evokes a series of responses which are defined as primary hemostasis. Initial events are modulated by exposed blood vessel components such as subendothelial matrix. Concomitantly, platelet adhesion and activation occur, but at this point proteins of the coagulation system are not directly involved. However, tissue factor may play an earlier role than previously anticipated (14). This sequence of events probably accounts for the clinical observation that the bleeding time in hemophilia is essentially normal, since it is not a platelet disorder *per se*.

As visualized in *ex vivo* studies, the vessel wall quickly retracts and platelets immediately adhere to subendothelium—especially the collagen component. vWF, both in the subendothelial matrix and from plasma, rapidly adsorbs to the site of damage and further mediates platelet adhesion through an interaction with the platelet glycoprotein Ib-IX-V receptor complex (5,15,16).

These events are accompanied by activation of gpIIb/IIIa on the platelet membrane (integrin $\alpha_{IIb}\beta_3$) (17,18). Activated platelets continue to spread and further adhere to the damaged vessel surface. They rapidly generate a releasate composed of a large variety of stored proteins, some of which serve as recruiting agents for platelets arriving at the injury site from the circulation. Platelet recruitment is the critical step in generation of a thrombus. The recruitment process ultimately promotes total occlusion of a vessel by the platelet thrombus. As mentioned, this early process is mediated in a major way by circulating vWF and by the vWF that is locally released from activated

platelets and endothelial cells. During high-shear-stress conditions, which occur in small vessels and in larger ones that have been partially occluded, vWF plays a critical role in generation of the platelet plug (15). In the setting described, continued local accumulation of activated platelets expands the physical dimensions of the thrombus through recruitment of additional platelets that arrive in the microenvironment (19,20).

The subendothelial collagen to which platelets initially adhere and on which they spread is a very strong agonist. (It meets this criterion because it induces about 65% platelet serotonin release *in vitro*.) The physiologic events initiated by platelet-collagen contact can be subdivided into four steps, as a working classification:

1. Biologically and biochemically active compounds that are mainly stored in intracellular platelet granules are secreted into the extracellular milieu. This is the classic platelet release reaction (5,16).
2. P-selectin, a platelet granule membrane glycoprotein, translocates to the platelet surface, where it mediates adhesion of activated platelets to neutrophils, monocytes, and certain lymphocyte subsets (21-24).
3. Activation of the platelet eicosanoid metabolic pathway commences with the appearance of free arachidonic acid (20 carbons, 4 double bonds), which is released from the platelet phospholipid compartment by the action of phospholipases (25-27). Free arachidonic acid is immediately oxygenated to form the labile, biologically active endoperoxide prostaglandin H_2 (PGH₂), a reaction catalyzed by prostaglandin-H endoperoxide synthase-1 (PGHS-1) (28). Isomerization of the endoperoxide to thromboxane A₂ is catalyzed by thromboxane synthase (29). Simultaneously, two compounds of unknown function are generated and released: 12-hydroxyheptadecatrienoic acid (HHTre) and malondialdehyde (MDA). Importantly, initial cyclooxygenation of arachidonate as catalyzed by PGHS-1, the prerequisite for formation of all of these compounds, is completely inhibited by aspirin. This medication irreversibly acetylates the "active site" serine residue of cyclooxygenase at position 530 (30).

Unprocessed free arachidonate escapes from platelets and is metabolized by cells in the surrounding milieu, such as endothelium, neutrophils, and monocytes (lymphocytes do not process arachidonate). This phenomenon results in biosynthesis of new, biologically active compounds and illustrates the phenomenon of transcellular metabolism (see later discussion) (13,26,31). Platelets also contain an aspirin-insensitive cytoplasmic enzyme, 12-lipoxygenase, which processes remaining free platelet arachidonate to 12-hydroxyeicosatetraenoic acid (12-HETE). This hydroxy acid is produced in great abundance after aspirin ingestion because the acetylated cyclooxygenase cannot process the accumulating free arachido-

- nate. The 12-HETE thus formed also leaves the platelet and metabolically interacts with other cells (e.g., neutrophils), leading to synthesis of new compounds (32).
4. The activated platelet undergoes a drastic transition in shape, from the smooth disk configuration of the resting state to a spiny sphere. This rearrangement results in more efficient platelet-platelet contact and adhesion. The shape change also produces a rearrangement of the platelet membrane phospholipoprotein compartment,

converting the platelet membrane into a highly efficient procoagulant surface (10). The shape change also permits optimal binding between the platelet membrane phospholipoprotein (not phospholipid) and activated coagulation factor X in the presence of activated factor V. Furthermore, the rearranged phospholipoprotein surface of the stimulated platelet activates factor VII via the extrinsic coagulation pathway. These events are summarized and depicted in Figure 6-2.

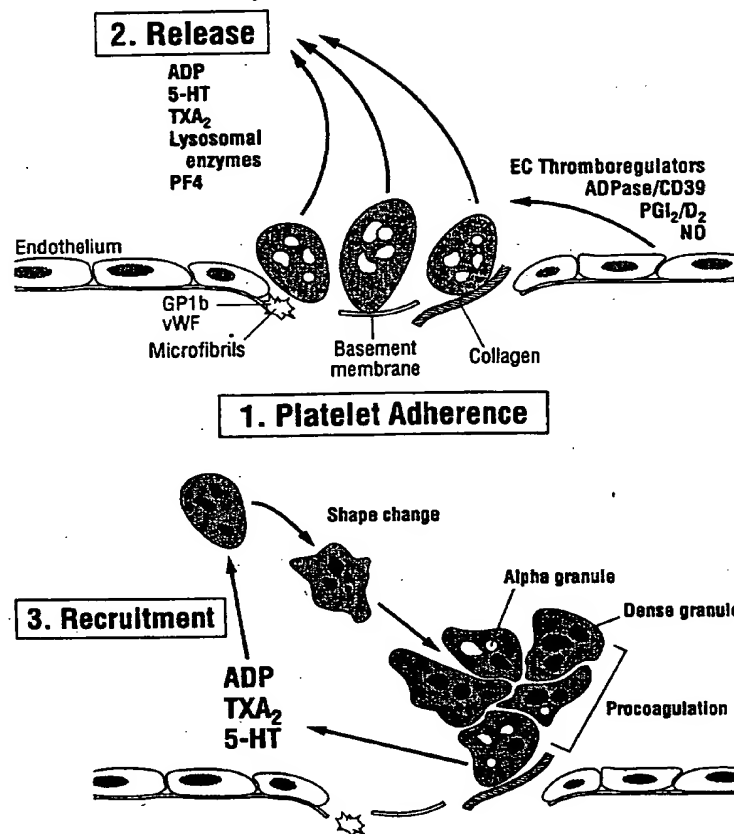


FIG. 6-2. Diagrammatic representation of the normal hemostatic process.

1, Platelet adherence begins. Exposure of subendothelium as a result of vascular injury causes immediate platelet adherence to collagen, basement membrane, and microfibrils in the presence of von Willebrand's factor and platelet glycoprotein Ib (GPIb).

2, Platelet release occurs. Collagen, a strong platelet agonist, induces release of adenosine diphosphate (ADP) and serotonin (5-HT) from dense granules. Several α granule proteins are also secreted. The eicosanoid thromboxane A₂ is formed enzymatically in platelets from released free arachidonate. Unmetabolized released arachidonate penetrates and is metabolized by other cell types in the microenvironment (33). Concomitant with adhesion and activation, endothelial cell defense mechanisms (thromboregulators) become functional and serve to limit the size of the thrombus.

3, Formation of the releasate initiates the recruitment phase of thrombin formation. ADP and thromboxane are the most important recruiting agents in the platelet releasate. Both 5-HT and thromboxane serve to induce vasoconstriction, thereby helping to consolidate the evolving thrombus and limiting the velocity of blood flow proximal to the thrombus. Components of the releasate serve to activate additional platelets arriving in the fluid phase. As the arriving platelets become activated, they undergo shape change and aggregate on the initial layer of adherent platelets. Phospholipoproteins on the platelet surface are now available for catalytic activation of proteins of the coagulation system.

The interactions described culminate in thrombin formation, an event that amplifies the initial activating steps described for collagen (see Fig. 6-2). Thrombin maximally activates platelets, which results in strong recruitment. The agonistic properties of thrombin are verified by the fact that this enzyme induces 90% serotonin release from platelets *in vitro*. The activating effect of thrombin induces maximal release, abundant eicosanoid

formation, and the appearance of fibrin in both the interstices and outer portions of the platelet thrombus (see Fig. 6-2). The end result is total occlusion of the blood vessel, which now contains a hemostatic plug in completely impermeable form. This secondary "consolidation phase" completes the hemostatic process. The platelet plug represents a consolidated mass of microscopic particles which, in conjunction with fibrin strands, must pos-

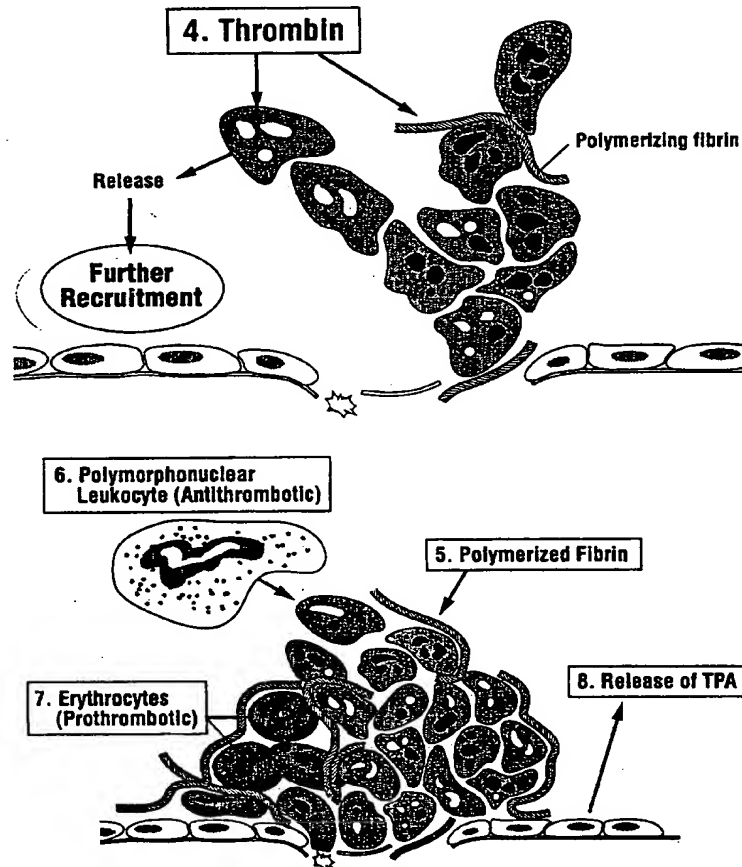


FIG. 6-2. *Continued*

4, Thrombin formation, the last stage of primary hemostasis, has important consequences that comprise the final steps of platelet thrombus development. Thrombin strongly induces further platelet activation, release and recruitment, as well as fibrin formation.

5, Fibrin strands begin to intercalate among activated platelets, and the entire thrombus becomes consolidated. The consolidated platelet thrombus is now virtually impermeable, and its multicellular nature is evident.

6, Polymorphonuclear leukocytes, which can be seen in close contact with platelets, serve an antithrombotic function in that they inhibit further platelet activation and recruitment (35).

7, Intact, metabolically viable erythrocytes are prothrombotic because they react to the presence of an activated platelet releasate with production of an unidentified material that increases platelet reactivity (34). Platelet-neutrophil contact is amplified by the adhesive glycoprotein, P-selectin, which interacts with its receptor on the neutrophil surface (22,23).

8, Formation of the platelet thrombus also signals initiation of the fibrinolytic process—that is, release of tissue plasminogen activator from endothelial cells (19).

sess enormous tensile strength to withstand the pressure of the circulation after it has occluded the blood vessel, either beneficially as a hemostatic plug or pathologically as a thrombus.

The Platelet Releasate: Components, Significance, and Methods of Study

Major components of the platelet releasate responsible for recruitment have already been discussed. From the standpoint of hemostasis and thrombosis, recruitment is the most important stage of platelet participation in vascular occlusion. Releasate constituents consolidate the thrombus and can also propagate it if intrinsic defense systems (thromboregulators) are overcome by the strength of the agonist (8,20).

In addition, as already described, components of the releasate such as free arachidonate and 12-HETE interact with other cells in the microenvironment by means of cell-cell interactions and transcellular metabolism. Released thromboxane serves as an agonist for vasoconstriction and platelet aggregation. It does not interact with cells other than platelets. ADP in the platelet releasate is metabolized by endothelial cell ecto-ADPase/CD39 (an example of thromboregulation). Calcium, originating from dense bodies, is prominent in the platelet releasate. This divalent cation may be important for calcium-requiring enzymes in the coagulation cascade or enzymes involved in crosslinking of deposited fibrin. Platelet-activating factor may promote platelet deposition in response to allergic injury (16).

Lysosomal enzymes are also secreted by platelets, although not in quantities comparable to leukocytes. Platelets are not inflammatory cells in the true sense. For example, in contrast to proinflammatory leukocytes, the platelet cannot generate an oxygen burst for production of superoxide. The platelet oxygen burst involves consumption of that element for oxygenation of arachidonic acid. Furthermore, platelets are not capable of engaging in true phagocytosis (i.e., they cannot produce a phagocytic vacuole). The contribution of platelets in inflammation is mainly in the form of substances they secrete. For example, platelets participate in the inflammatory process through transcellular metabolism of eicosanoid precursors and intermediates (13,25,33).

Platelet-released heparatinase cleaves surface glycosaminoglycans on endothelial cells to produce a fragment with antiproliferative properties. The transglutaminase, factor XIII, catalyzes peptide bonds between the γ -glutamyl residues and ϵ -amino groups of lysines to form stable crosslinks between fibrin in the interstices between platelets and fibrin surrounding the clot. Factor XIII can also cross-link fibronectin and α_2 -antiplasmin to fibrin. Factor XIII is the only released cytoplasmic platelet protein; all others originate from granules. Platelet-derived growth factor (PDGF), which is secreted

from α granules, modulates growth and patterns of gene expression in cells of the vessel wall (11,12). PDGF also promotes the smooth muscle proliferation that occurs as a consequence of platelet-vessel wall interactions. Receptors for PDGF are transmembrane tyrosine kinase-type molecules. The connective tissue-activating peptide III is thought to be a precursor of β -thromboglobulin and is probably involved in fibroblast proliferation. It is structurally similar to platelet factor-4, and both belong to a protein class involved in growth control and the inflammatory response.

Transforming growth factor- β (TGF- β) is of platelet origin and is an important agonist for synthesis of extracellular matrix molecules and their receptors. TGF- β has a broad spectrum of effects on cell proliferation, being inhibitory in some instances and mitogenic in others. Platelets are the major source of thrombospondin, which plays a role in platelet aggregation, activation of TGF- β , and angiogenesis. Endothelial and smooth muscle cells also produce thrombospondin (16). Proteins involved in the coagulation cascade and in cell adhesion are also components of the platelet releasate, having originated in platelet α granules. As mentioned, plasma proteins are taken up by developing megakaryocytes and stored in α granules during maturation in the marrow. Examples are albumin and IgG, which can be detected in platelet α granules. However, their concentration is much lower than in plasma, so they probably were not biosynthesized in the developing megakaryocyte. These findings have confounded measurements of platelet-associated IgG in patients with thrombocytopenia (16).

In contrast, the quantity of factor V in platelet α granules is higher than can be explained by uptake from plasma. Platelet factor V is critical for assembly of coagulation proteins on the stimulated platelet surface. The finding of protein S in platelets is interesting because it is a cofactor for activity of activated protein C. Plasminogen activator inhibitor 1 (PAI-1), which is released from activated platelets, may be important for control of fibrinolytic activity in the microenvironment of a thrombus (16).

An unexpected component of the platelet releasate is the β -amyloid precursor protein (APP). This α -granule protein is a precursor of about 40 peptide residues identified in amyloid deposits in brain tissue of patients with Alzheimer's disease. APP belongs to the protease inhibitor family and can be released by proteolytic cleavage. As with other contents of the platelet releasate, one can ask whether APP plays a direct or indirect role in platelet function (16).

P-selectin is not a component of the platelet releasate. It is an α -granule membrane protein which, as mentioned, translocates to the platelet surface after activation (21-23). P-selectin binds specific carbohydrates and belongs to the same family as E- and L-selectin, which play a role in adhesive processes between leukocytes. P-

selectin itself controls interactions of monocytes and neutrophils with platelets. Selectins are prototype systems for cell-cell interactions resulting in leukocyte recruitment in both hemostasis and thrombosis, as well as in the inflammatory response.

Adhesive proteins are also found in releasates from platelet α granules. Platelet vWF is synthesized in megakaryocytes and concentrated in platelets. The platelet form of vWF contains larger multimers, which are hemostatically more effective than smaller multimers. Fibronectin in platelets is present in alternatively spliced forms that are not found in plasma. This indicates that platelet fibronectin may play a role in matrix assembly. Vitronectin is found in low concentrations in platelets. PAI-1 binds to vitronectin, and both proteins are released in complex during platelet activation (16).

Platelet Activation and Recruitment as Studied in the Laboratory

The importance of the platelet releasate as a recruiting agent for other platelets and as a transport system for secreted platelet components that interact with other cells in the circulation has repeatedly been emphasized. If platelet activation and recruitment could be studied separately, the information would be more specific than that obtained from cocubation systems, as was demonstrated by Santos and Valles (34-36). Their novel system is demonstrated in Figure 6-3. Platelets alone, or platelets and other cells in combined suspension, are incubated in the presence of stirring to promote cell-cell contact. An agonist is added to this suspension, which is then inverted three times for 10 seconds. During the next 50 seconds, the tube is centrifuged and the cell-free releasate is removed for further testing. Components of the releasate are analyzed biochemically, and the releasate itself is used as an agonist for platelet-rich plasma (also stirred). By this method it is possible to separate and analyze the fluid phase of activated platelets for specific proteins and

other released, biologically active compounds such as ADP, thromboxane, serotonin, and PDGF.

This system of study has revealed both basic and clinically applicable information. For example, on exposure to the fluid phase of activated platelets, erythrocytes release an aspirin-insensitive substance that promotes platelet reactivity. Interesting concepts have emerged from these results. Low-dose aspirin administration to patients with vascular diseases loses its protective effect with time. The low-dose aspirin must be supplemented with a therapeutic dose (325 mg) every 2 weeks (36). Neutrophils inhibit platelet reactivity when exposed to the releasate from activated platelets. Therefore, neutrophils accumulating at the site of a thrombus may exert a protective effect against its propagation (see Fig. 6-2) (34,35).

Role of G Proteins in Platelet Activation

The G protein group consists of an homologous array of guanine nucleotide-binding regulatory proteins. These proteins modulate interactions between receptors on the cell surface and effector molecules in the cell. The effectors are enzymes that generate second messages and also control movement through ion channels. In platelets, G proteins regulate adenylyl cyclase, phospholipase C, and phospholipase A_2 . The G proteins are heterotrimeric and consist of α -, β -, and γ -subunits. The α -subunits are the most diverse and account for most of the activities of the G protein group. In resting cells, the α -subunit is bound to guanosine diphosphate (GDP). This complex associates with $\beta\gamma$ -subunits. When the G protein instead binds guanosine triphosphate (GTP), activation results (Fig. 6-4). Receptor interaction with agonists such as thrombin is most efficient when G protein is in the heterotrimeric form. Platelet receptors that couple to G proteins include thrombin and the protease-activated receptor-2 (PAR-2). Others include those for epinephrine, thromboxane A_2 , vasopressin, and platelet-activating fac-

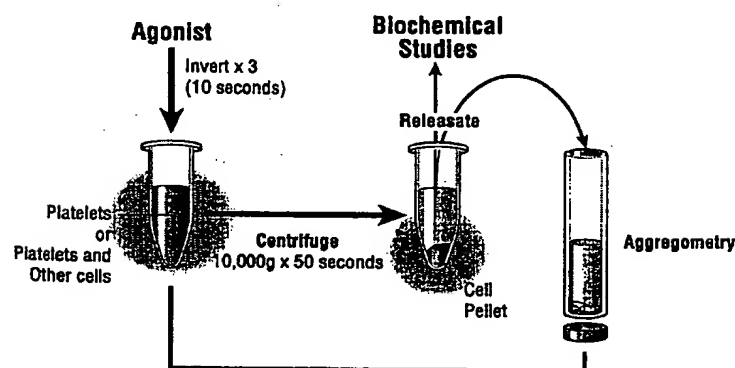
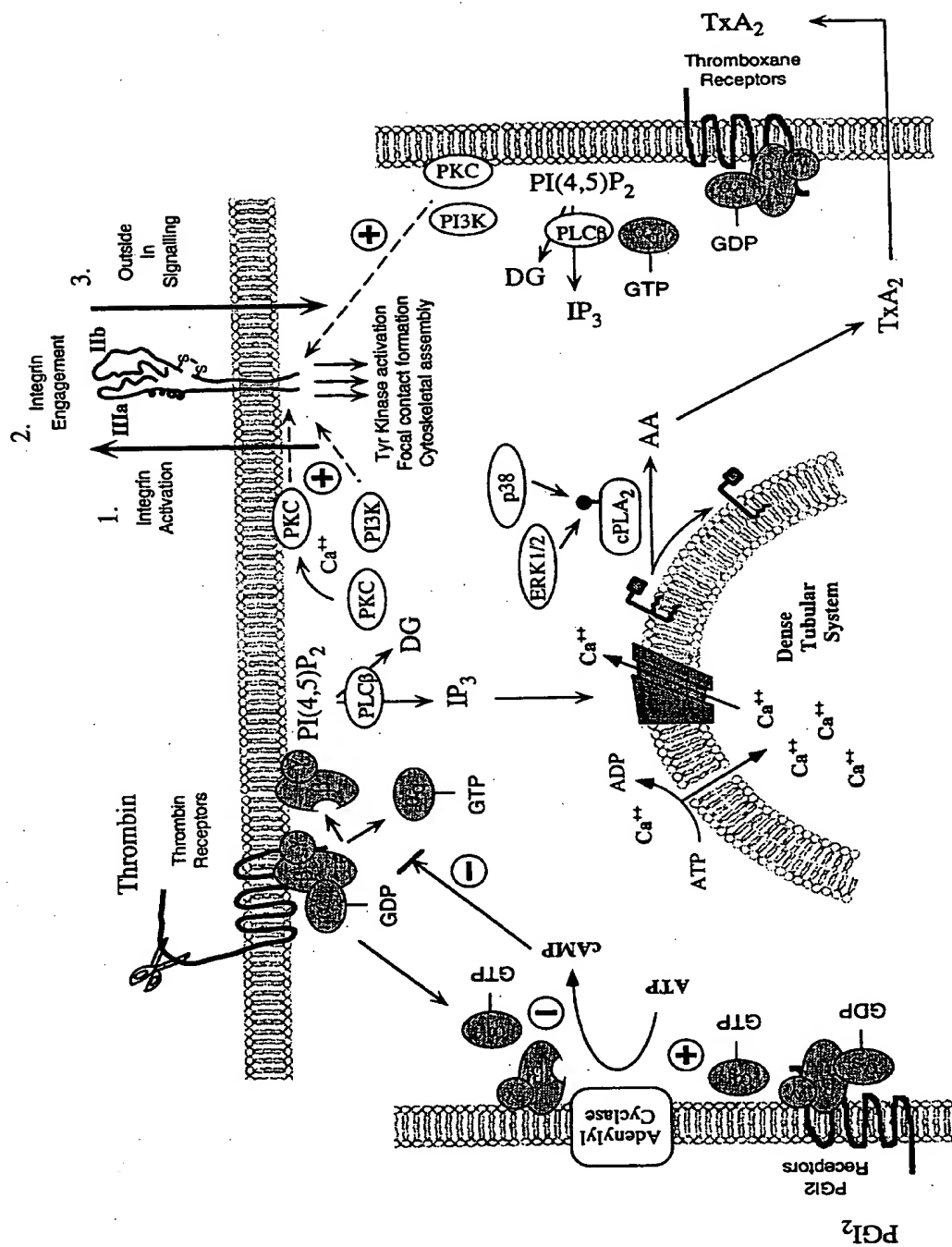


FIG. 6-3. System for separate evaluation of platelet activation (generating system, *left*) and recruitment (assay system, *right*). An agonist is added to the generating system in order to activate platelets alone or platelets plus other cells. A cell-free releasate is obtained after centrifugation of the generating system. This releasate is transferred to the assay system (platelet-rich plasma) for assessment of proaggregatory activity (recruiting properties) of the releasate and for biochemical studies to measure activation. These include studies of serotonin (5-HT) release, arachidonic acid release and metabolism, and agonistic or inhibitory properties of combined cell suspensions that were exposed to an agonist (34-36).



tor. When thrombin binds to and cleaves its receptor's N-terminus between residues Arg⁴¹ and Ser⁴², a "tethered ligand" domain is exposed (i.e., the amino acid string, SFLLRN). It was research on thrombin receptors and PAR-2 that first led to the concept that a cleaveable cell surface receptor could initiate intracellular signaling. In the future, other proteases that can activate cells in a similar manner, through receptors on the cell surface, should be identified (37,38).

In platelets, adenylyl cyclase catalyzes formation of cyclic adenosine monophosphate (cAMP) from ATP. This promotes inhibition of platelet reactivity by blockade of calcium mobilization. Different types of G proteins can regulate adenylyl cyclase. Prostacyclin (PGI₂) from endothelial cells is another stimulus for adenylyl cyclase through the G protein G_s. Thrombin inhibits cAMP formation through the action of one of the forms of G_i that exist in platelets (37). Signal transduction during platelet activation is summarized and depicted in Figure 6-4.

Phosphorylation and Dephosphorylation Systems in Platelets

Levy-Toledano et al. (39) outlined several phosphorylation pathways occurring in the course of platelet activation:

1. Thrombin activates phospholipase C_β in a reaction stimulated by the heterotrimeric G_i subunit protein that is coupled to the seven-transmembrane domain receptor of thrombin. Phospholipase C acts on phosphatidylinositol 4,5-bisphosphate (PIP₂) in the mem-

brane, leading to synthesis of the second messenger inositol-1,4,5-trisphosphate (IP₃), which mobilizes intracellular calcium, and diacylglyceride, which activates protein kinase C. The latter catalyzes phosphorylation of substrates at serine/threonine residues, such as myosin light chain (20 kd) and pleckstrin (47 kd). Hydrolysis of PIP₂ results from stimulation of phospholipase C_γ, which requires tyrosine kinase activity but is independent of G protein.

2. Thrombin increases levels of phosphotyrosines on several proteins. This function depends on integrin α_{IIb}β₃ involvement and platelet aggregation. (Blockade of fibrinogen binding inhibits tyrosine phosphorylation). When platelets are tyrosine phosphorylated, there is activation and translocation of several nonreceptor protein-tyrosine kinases, such as the SRC family kinases (60 kd), SYK (72 kd), and FAK (125 kd). SYK also contains two SRC homology-2 (SH2) domains and one SH3 domain, which are involved in regulation of protein-protein interactions in platelets.
3. Platelet stimulation and activation of surface receptors results in activation of phosphoinositide 3-kinase. This important enzyme catalyzes phosphorylation of inositol phospholipids at the 3-position of the inositol ring (40,41). The phosphoinositide 3-kinase in platelets catalyzes formation of phosphatidylinositol 3,4-P₂ and phosphatidylinositol 3,4,5-P₃. These are important second messengers for platelet function (42).
4. Mitogen-activated protein kinase (MAP kinase) is an important signaling molecule that transfers receptor

FIG. 6-4. Pathways of signal transduction in response to platelet activation. When agonists bind to their receptors on the platelet membrane, several intracellular second messengers are activated. Phosphatidylinositol 4,5-bisphosphate (PIP₂) is hydrolyzed by phospholipase C_β (PLC_β) to generate inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DG). PLC_β is activated by G protein, probably G_q, derived from G_i, in a manner that is sensitive to pertussis toxin. PLC_β is also activated in a pertussis toxin-resistant manner by a separate G protein, G₁₂ or G₁₃ or both. IP₃ releases calcium from the dense tubular system, resulting in an increase in free calcium concentration in the platelet cytosol. DG activates protein kinase C, which induces the platelet release reaction (inside-out signaling) and exposure of the fibrinogen receptor by reorientation of the gpIIb/IIIa complex. Fibrinogen binding to the gpIIb/IIIa complex (integrin engagement), is followed by outside-in signaling such as tyrosine kinase activation (43). The rise in cytosolic free calcium promotes arachidonic acid release as catalyzed by the cytosolic form of phospholipase A₂ (cPLA₂), culminating in thromboxane A₂ formation and release. Phospholipase A₂ may also be activated at the cell membrane by G_q, originating from one or more G proteins. The thromboxane A₂ formed from released arachidonate interacts with receptors on the platelet membrane, inducing further platelet activation. During the activation process, tyrosine kinases such as members of the SRC family are activated and phosphorylate a large variety of platelet proteins, many of which have not been precisely identified. In addition, focal contact formation occurs, as does cytoskeletal rearrangement (43). Tyrosine activation in platelets occurs at a later time than fibrinogen receptor exposure and platelet aggregation. The thrombin receptor is coupled to G proteins, as are the thromboxane and prostacyclin (PGI₂) receptors.

Platelet activation is always accompanied by a fall in cyclic adenosine monophosphate (cAMP). Elevations in cAMP block calcium mobilization, thereby inhibiting platelet reactivity (37). (Courtesy of Dr. Lawrence F. Brass.)

signals to multiple pathways. These pathways are used for signaling throughout the platelet cytoplasm. The MAP kinase pathway is stimulated by platelet agonist exposure. In this pathway, low-molecular-weight GTP-binding proteins such as RAS play a role.

Platelet protein kinases and phosphatases have an integrative activity and amplify signals induced by multiple receptors at the cell surface. Interactions among these kinases and phosphatases remain to be determined in detail, and their delineation will increase our comprehension of platelet phosphorylation and dephosphorylation systems. New information should emerge from gene knockout experiments, use of antibodies to the kinases and phosphatases, and development of highly specific inhibitors of individual kinases or phosphatases (39).

To summarize, on platelet activation an intracellular metabolic event (possibly protein kinase C activation) involves $\alpha_{IIb}\beta_3$, inducing its ability to engage fibrinogen on the extracellular domain of this receptor. This initiates an "outside-in" integrin signaling event through $\alpha_{IIb}\beta_3$, which is critical for platelet reactivity. Signaling proteins such as SYK and FAK become increasingly tyrosine phosphorylated, calcium is mobilized, calpain is activated, and the cytoskeleton undergoes reorganization (39) (see Fig. 6-4).

Integrin Signaling in Platelet Function

The integrin class of receptors is required for development of vascular and hematopoietic cells, angiogenesis, cell migration in response to injury, and assembly of extracellular matrix. The platelet integrin $\alpha_{IIb}\beta_3$ is mandatory for hemostasis (43). The outstanding work of Collier has led to development of a therapeutic agent that inhibits $\alpha_{IIb}\beta_3$ (17,18).

Integrins are composed of noncovalent $\alpha\beta$ heterodimers. Subunits consist of a large NH₂-terminal extracellular domain, a single domain that spans the membrane, and a COOH-terminal cytoplasmic tail. Numerous α - and β -subunits have been cloned, and there are at least 20 different $\alpha\beta$ pairings in various tissues. Although integrins were originally thought to function solely as adhesive proteins, it is now recognized that they play an equally important role as receptors in signaling. Shattil and Ginsberg (43) defined integrin signaling as the ability of these receptors to transmit biochemical messages in both directions across the cell surface.

Integrins can also regulate gene expression, cell growth and differentiation, and survival through the mechanism of outside-in signalling. Ligand-occupied and clustered integrins control both cell shape and organization of the cytoskeleton. In addition, ligand-engaged integrins can generate a broad spectrum of biochemical signals. Integrin-triggered reactions include activation of protein-tyrosine kinases such as pp60^{SRC}, pp125^{FAK}, and

pp72^{SYK}. They can also activate phosphatidylinositol 3-kinase and MAP kinases. These activation steps are initiated by means of other agonist receptors, such as those that interact with growth factors and cytokines. The initial focus of integrin signaling is topographic, on locations of the cell surface where cell-cell and cell-extracellular matrix contact takes place. These reactions involve alterations in cell shape, polarization, and motility—all of which are classified as "anchorage-dependent" changes (43).

Integrins have the capacity to promote extracellular effector responses as they undergo ligand engagement during activation. Binding of ligands is regulated by signaling mechanisms in the cell, a process called *integrin activation* or "inside-out signaling." By this means, intracellular signals are translated into extracellular activation.

The initial binding of soluble fibrinogen or vWF to $\alpha_{IIb}\beta_3$ is controlled by affinity modulation. When affinity is modified, it is implied that a structural change has occurred in the integrin heterodimer, resulting in stronger ligand binding. Various affinity states have been studied in integrins of several β classes, using soluble ligands that specifically induce cell activation. The different affinity states probably reflect conformational changes in and between receptor subunits. These ultimately influence the shape or accessibility of the ligand-binding interface.

Antithrombotic medications currently in use probably function by regulating the capacity of platelet-signaling mechanisms to initiate conformational changes in $\alpha_{IIb}\beta_3$. A high-affinity state may not always be beneficial. When there is high integrin affinity, migration can be blocked if substrate and integrin densities are also elevated. In addition, the ability of cells to assemble a fibronectin matrix is regulated by the activation state of integrins (43).

Integrins cluster or multimerize on the plasma membrane (44). Clusters of ligand-occupied integrins in adherent cells such as platelets, endothelial cells, and vascular smooth muscle can be observed by light microscopy. These clusters form small, focal complexes that assemble during activated platelet filopodial extension. Larger focal adhesions connect with actin stress fibers and assemble during late stages of platelet spreading. Filopodia and focal adhesions are regulated by the *rho* group of GTPases; they form a link between *rho* signaling and integrin function. Focal complexes and focal adhesions contain a broad spectrum of signaling molecules and cytoskeletal proteins. Assembly and disassembly of these complexes is essential for platelet reactivity and vascular cell migration.

The cytoplasmic domains of integrins are critical for integrin signaling because they interface with signaling components of the cell. Most integrin tails range from about 20 to 70 amino acid residues in size. If membrane-distal sequences of β cytoplasmic tails are mutated or truncated, integrin-initiated signaling and cytoskeletal organization are disrupted.

A large number of proteins can bind directly to integrin cytoplasmic tails (some to α tails, others to β tails) (43). Although many binding proteins have been identified, the role of this binding in cell function is unknown. Some binding proteins are part of the cytoskeletal compartment (α -actinin, filamin, talin), and others have kinase activity (pp125^{FAK}, integrin-linked kinase [ILK]). Some have guanine nucleotide exchange activity (cytohesin-1), and others function as adaptors (paxillin). Calreticulin binds to the membrane-proximal portion of α cytoplasmic tails. This protein is found in several subcellular compartments and may be involved with other adhesion receptors and cytoskeletal proteins. Calreticulin-null embryonic stem cells do not participate in integrin-mediated cell adhesion and calcium influx.

Integrin signaling also involves association of integrins with other transmembrane proteins. β_3 integrins interact with the integrin-associated protein, CD47. This could function in regulation of neutrophil phagocytosis in response to ligand agonists. CD9, CD63, and CD81 belong to the tetraspanin class of transmembrane proteins. When detergent extracts are prepared from some cells, the tetraspanins coprecipitate with integrins. These associations can be promoted by antibody crosslinking of integrins. Therefore, tetraspanins may be involved in regulation of integrin signaling and cell migration. Tetraspanins and integrins are also physically associated with phosphatidylinositol 4-kinase. This suggests a relation between integrins and phosphatidylinositol metabolism (43,44).

In some instances, β_1 integrins promote cell growth, and in others, they promote differentiation. Correlations have been made between growth promotion and the ability of β_2 integrins to activate the MAP kinase pathway through the adaptor, SHC. Formation of physical complexes with SHC correlates with associations between extracellular or transmembrane domains of the integrin α -subunit and caveolin. The latter protein may provide a supporting framework for signaling molecules (44).

In summary, the adhesive and signaling functions of integrins are very important for control of the reactivity of platelets and inflammatory cells. Specific roles have been identified for integrin signaling events in angiogenesis, cell migration during development, hemostasis, inflammation, and wound repair. Unwanted or uncontrolled integrin signaling is probably involved in the pathogenesis of occlusive vascular diseases and atherosclerosis, as well as angiogenesis in diseases such as diabetic retinopathy (43,44).

THROMBOREGULATION

Thromboregulation is defined as a group of processes by which circulating blood cells and cells of the vessel wall interact to regulate development of a thrombus. Most thromboregulatory activities occur in the setting of

cell-cell interactions. The reactions are biochemical in nature and can result in formation of biologically active metabolites that could only have arisen through interactions between heterogeneous cell types in the vasculature (5,13,19,33,45,46).

Thromboregulators are mainly responsible for maintenance of blood fluidity *in vivo*. They prevent or reverse platelet accumulation, activation of coagulation factors, and the fibrin formation that these processes produce. These defense systems can be overwhelmed by vascular injury in the form of a fissured or fractured atherosclerotic plaque, disturbances in blood flow, or inactivation or destruction of cell-associated or fluid-phase thromboregulators. The fibrinolytic system also participates in thromboregulation by preventing excessive fibrin formation and enhancing its removal (5,8).

Currently known thromboregulators can be classified according to their chronologic modalities of action in relation to thrombin formation (Table 6-1). For example, the protein C-protein S natural anticoagulant system is operative *after* thrombin has formed at an injury site. Thrombin then enters the fluid phase of the microenvironment and binds thrombomodulin on the proximal endothelial cell surface. In arterial thrombosis, thromboregulatory mechanisms are nullified by the agonistic effect of injured tissue and tissue factor (14). Alternatively, these thromboregulatory mechanisms fail and the natural proclivity toward fibrin formation at the site of injury escapes regulation. The ADPase/CD39 system is operative very early in the hemostatic/thrombotic cascade.

Cell-Cell Interactions and Transcellular Metabolism

When the morphology of evolving thrombi was initially studied by light and electron microscopy, erythrocytes, neutrophils, and platelet components could be seen in close proximity (Fig. 6-5). These associations were initially interpreted as passive in nature. Cells other than platelets were not thought to be biochemical participants in the formation of a thrombus. However, both *in vitro* evidence and clinical correlations now support the con-

TABLE 6-1. Classification of vascular thromboregulators

Early thromboregulators (inhibit events preceding thrombin formation)
Nitric oxide (NO)
Eicosanoids (prostacyclin, PGD ₂)
Endothelial cell ecto-ADPase/CD39
Late thromboregulators (exert effects after thrombin formation)
Antithrombin III
Endothelial cell/heparan proteoglycans
Tissue factor pathway inhibitor (TFPI)
Thrombomodulin-protein C-protein S pathway
Proteins of the fibrinolytic system

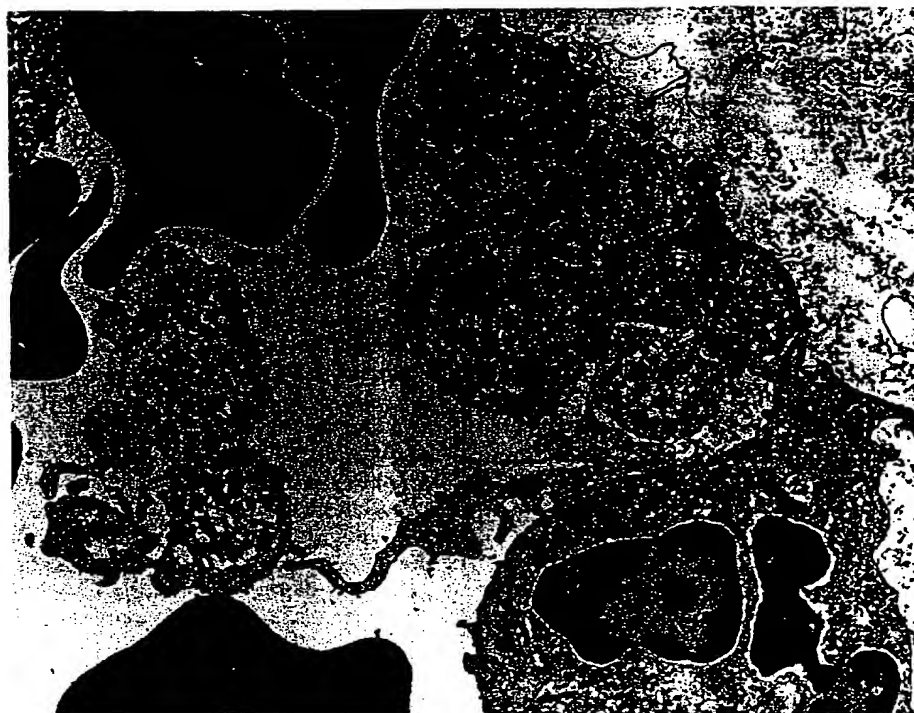


FIG. 6-5. Electron micrograph of an evolving thrombus after addition of adenosine diphosphate to whole blood. Platelets have partially degranulated and are in the process of forming an aggregate. Erythrocytes can be seen in close proximity and in some areas actually making contact with the platelet surface. Such erythrocytes, when exposed to activated platelets, release material that promotes platelet reactivity. A neutrophil can be seen in the lower right portion of the figure, also making contact with activated platelets. Neutrophils under these circumstances release material that inhibits platelet reactivity. These interactions are described in detail in the text and are comparable to sections 6 and 7 of Figure 6-2. (Courtesy of Dr. Dorothea Zucker-Franklin, Department of Medicine, New York University School of Medicine, New York, New York.)

cepts already mentioned: erythrocytes enhance platelet activation and recruitment (34,36), whereas neutrophils inhibit platelet reactivity and may play a role in limiting the size and extent of a thrombus (5,8,13,19,27,31,35).

These cell-cell interactions occur in addition to those described for platelets and endothelial cells (45-47). Besides direct metabolic interchange between cellular components of a thrombus, there is another form of cell communication via the eicosanoid pathway, known as *transcellular metabolism* (26,32,33,45,48). Studies of this pathway have been elegantly developed by Serhan et al., especially with regard to the lipoxin group of autacoids (25,26,48).

Initial studies of eicosanoids focused on arachidonic acid mobilization and metabolism in single cell types; this is summarized in Figure 6-6. It is now known that eicosanoid metabolism also takes place between different cell types in close proximity. Reactive eicosanoid precursors and reactive intermediates can traverse the fluid phase between neighboring cells, thereby giving rise to

biologically active metabolites with properties different from those normally synthesized by either cell alone. Cell-cell interactions and transcellular metabolism are important modalities for the amplification and generation of eicosanoid-derived inflammatory mediators, especially those catalyzed by lipoxigenase enzymes in various cells. All of these lipoxigenase-driven reactions are insensitive to aspirin and, in fact, are frequently enhanced after aspirin ingestion. These systems are of potential significance for hemostasis, thrombosis, and the inflammatory response (25,27,32,33,45). My colleagues and I have proposed a working classification of cell-cell interactions and transcellular biosynthesis in the eicosanoid pathway (49); this is shown in Table 6-2.

An example of type IA interactions is the utilization of platelet-derived endoperoxides by endothelial cells that have been pretreated with aspirin. This results in the formation of PGI_2 from released platelet endoperoxides (47). In type IB interactions, a cell cannot produce a precursor endogenously but can further process a precursor

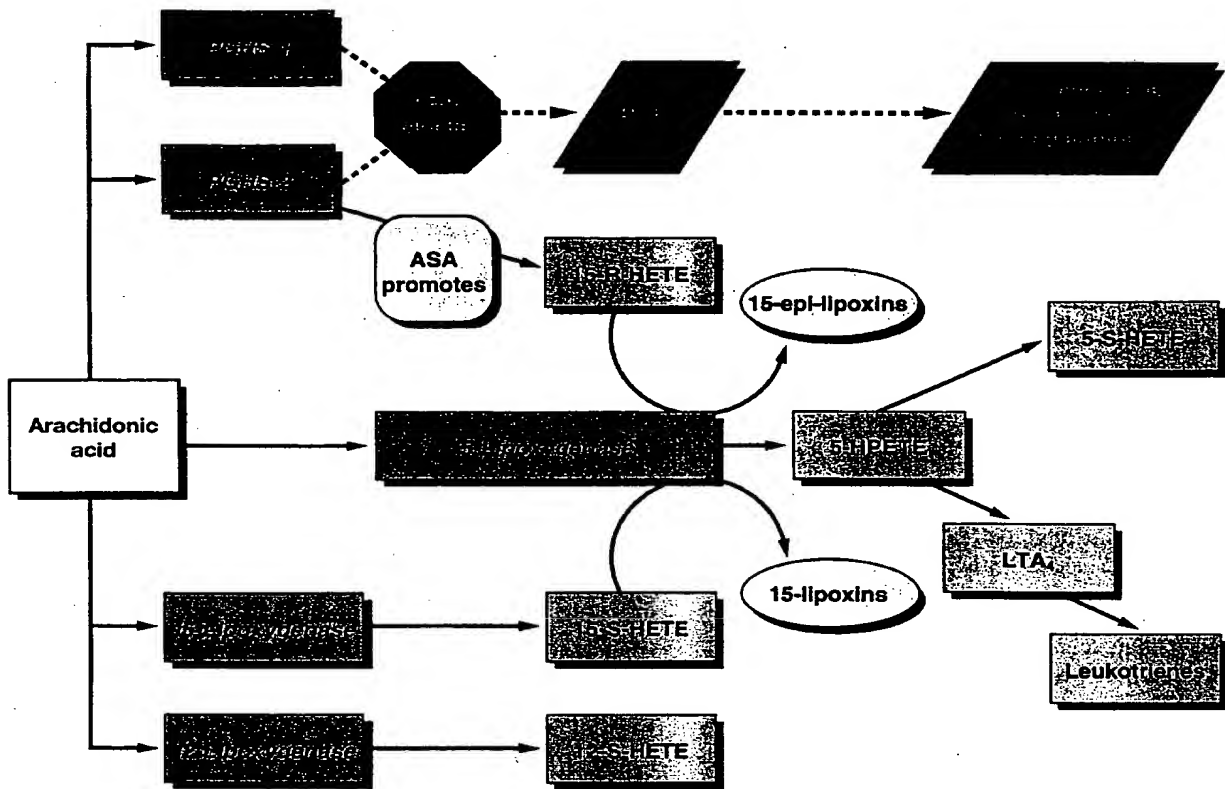


FIG. 6-6. In response to inflammatory or prothrombotic stimuli, arachidonic acid, an essential fatty acid, is released from cell membrane phospholipids. It is metabolized by two classes of oxygenases: prostaglandin-H endoperoxide synthases (PGHS-1 and -2, also known as cyclooxygenases) and lipoxygenases. PGHS-1 and -2 catalyze the insertion of two molecules of oxygen into arachidonic acid to form prostaglandin endoperoxide (PGH₂), which is then converted to thromboxane A₂ in platelets, prostacyclin (PGI₂) in endothelial cells, and other prostaglandins. Aspirin (ASA) completely blocks formation of PGH₂ and its metabolites by irreversibly acetylating serine in the active site of PGHS-1 and -2. However, in contrast to PGHS-1, PGHS-2, an independent gene product, retains its capacity to add one molecule of oxygen to the C15 position of arachidonic acid, even when acetylated. Aspirin-treated cells induced by inflammatory stimuli to express PGHS-2 therefore can produce the monohydroxy fatty acid (15*R*)-hydroxyeicosatetraenoic acid, or (15*R*)-HETE. The second class of oxygenases (5-, 12-, and 15-lipoxygenases) are insensitive to ASA. They catalyze the production of an abundance of the monohydroxy fatty acids (5*S*)-HETE, (12*S*)-HETE, and (15*S*)-HETE. When the enzyme activity of PGHS-1 and -2 is inhibited by ASA, more arachidonic acid is available for lipoxygenase metabolism (aspirin shunt). Various types of cells interact to metabolize (15*S*)-HETE and other lipoxygenase metabolites (by transcellular metabolism) to products including the lipoxins (25). (Modified from ref. 66, with permission.)

obtained from another stimulated cell. For example, the erythrocyte can transform leukotriene A₄, obtained from a stimulated neutrophil, to leukotriene B₄ (50). In addition, platelets can generate leukotriene C₄ from leukotriene A₄ (51). This reaction is of clinical significance because leukotriene C₄ production is not blocked by aspirin and this autacoid is a vasoconstrictor (13,19).

The type IIA reaction occurs when neutrophils and platelets are exposed to a common agonist, such as calcium ionophore A23187. Released platelet 12-HETE is used by activated neutrophils for production of (5*S*,12*S*)-

diHETE (45). Of critical importance for understanding of type IIA reactions was the elucidation by Serhan of 5-lipoxygenase-initiated lipoxin synthesis (25,48): the 5-lipoxygenase from activated neutrophils and the 12-lipoxygenase from activated platelets promote the formation of lipoxins A₄ and B₄ (52). Just as neutrophils can convert platelet-derived 12-HETE into (5*S*,12*S*)-diHETE, so 5-HETE from activated neutrophils can be converted to (5*S*,12*S*)-diHETE by activated platelets. This interaction diverts neutrophil production of the proinflammatory molecule leukotriene B₄. These dihydroxy acid

TABLE 6-2. Generation of new mediators via cell-cell interactions and transcellular metabolism in the eicosanoid pathway

Type I: Common eicosanoid precursors can be shared by different cells.	
Type IA:	In addition to generating its own precursor, a cell can obtain the same compounds from another; more end product is thereby synthesized.
Type IB:	A cell cannot synthesize a precursor endogenously but can obtain it from a stimulated neighboring cell and use it for novel eicosanoid synthesis.
Type II: A cell can transfer an eicosanoid from a neighboring cell into a new metabolite that neither cell alone can synthesize.	
Type IIA:	Both cells are activated by a common agonist.
Type IIB:	An activated cell produces an eicosanoid that an unstimulated cell in proximity can use for generating a new metabolite.
Type III: An intermediate or eicosanoid generated by one cell can serve as an agonist or inhibitor for biosynthesis of a different type of eicosanoid from a neighboring cell.	
A leukotriene can serve as agonist for thromboxane production.	

eicosanoids can serve as antiinflammatory compounds by an indirect mechanism (26).

In type IIB reactions, only one of the two cell types under study is activated. For example, if thrombin or collagen is added to a combined suspension of neutrophils and platelets, released platelet 12-HETE will be metabolized by the unstimulated neutrophils to 12,20-diHETE. (Thrombin and collagen do not activate neutrophils.)

In type III cell-cell interactions, eicosanoids themselves serve as agonists for production of other eicosanoids in a different cell type. For example, leukotrienes can serve as agonists for thromboxane release in perfused lung preparations (53).

Such developments in eicosanoid research have provided answers *in vitro* and *in vivo* for phenomena that could not be explained previously (27) (see Table 6-2). Furthermore, these reactions demonstrate a direct relation between thrombosis and the inflammatory response (25). As a result, comprehension of the involvement of eicosanoids in host response systems has been greatly amplified. Novel eicosanoids have been discovered, several receptors have been identified and cloned, and it is now understood that these lipid mediators are critical for signal transduction and cell-cell communication (25). Relations among precursors, intermediates, and end products in the eicosanoid pathways are depicted in Figure 6-6.

Blockade of Platelet Activation and Recruitment by Endothelial Cell Ecto-ADPase/CD39

As emphasized previously, appreciation of the importance of vascular cell-cell interactions and transcellular metabolism in thrombosis and inflammation has markedly increased in recent years (13,19,26,31). This is particularly pertinent with regard to platelets and endothelial cells. My colleagues and I currently hypothesize that endothelial cells control platelet reactivity by at least three mechanisms (see Table 6-1): a cell-associated

ecto-ADPase system and two fluid-phase reactants—eicosanoids such as PGI₂ and the relaxing factor nitric oxide (NO), which is generated by endothelium (54,55).

In previous work, we demonstrated inhibition of platelet aggregation by PGI₂ synthesized by aspirin-treated endothelial cells from platelet endoperoxides (47). We subsequently performed experiments in which NO production was neutralized by hemoglobin and both platelets and endothelial cells were treated with aspirin, resulting in total blockade of PGI₂ production. Biochemical and functional data indicated that these aspirin-treated, NO-deficient endothelial cells were actually inhibiting platelet function via a mechanism involving metabolism of ADP with consequent loss of this nucleotide's ability to induce platelet activation and platelet recruitment (56). We found that when aspirin-treated, washed platelets were stimulated by agonists in the presence of aspirin-treated endothelial cells, platelet aggregation and recruitment were inhibited (Fig. 6-7):

To establish whether ADPase activity was present on cultured human umbilical vein endothelial cells (HUVEC) and could account for their platelet-inhibitory properties, biochemical and functional assays were developed (56). Carbon 14-labeled ADP was incubated with aspirin-treated HUVEC for increasing periods. After removal of the cells, supernatants were examined for content of residual [¹⁴C]ADP and its metabolites. In addition, the platelet-aggregating potential of supernatant fluid containing unmetabolized [¹⁴C]ADP was examined. HUVEC induced a progressive decrease in [¹⁴C]ADP concentration, which was paralleled by a loss of proaggregatory activity of the supernatant fluid. Thin-layer chromatography was performed with radiographic scans of the metabolites of [¹⁴C]ADP after 5, 10, and 30 minutes of incubation with aspirin-treated HUVEC suspensions. At 5 minutes, ADP had fallen to account for 51% of total nucleotides, nucleosides, and bases. In addition, AMP (28%) and inosine (13%) were identified, together with trace quantities of adenosine, hypoxanthine, and

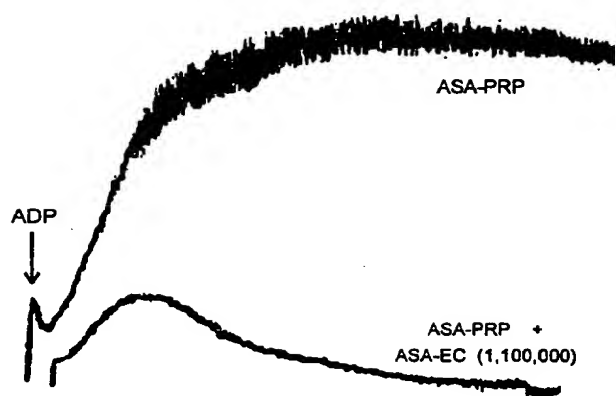


FIG. 6-7. Platelet-rich plasma from a donor who had ingested aspirin (ASA-PRP) was fully aggregated by adenosine diphosphate (ADP). In contrast, when this plasma was stimulated in the presence of 10^6 aspirin-treated endothelial cells (ASA-EC), the inhibited aggregation curve demonstrated a brief ascending limb, followed by reversal to baseline. This pattern of reversibility was reminiscent of previous experiments in which ADP released from platelets by agonists was intentionally removed by addition of purified apyrase. Subsequently, the endothelial cell membrane component responsible for the inhibition was shown to be an apyrase identical to CD39 (46,56).

adenine. After 30 minutes, inosine (85%) was the major [^{14}C]ADP metabolite and ADP itself was virtually absent. This absence of ADP correlated with total loss of aggregatory activity of the aspirin-treated endothelial cell supernatant. These studies indicated that ADP hydrolysis by endothelial cells is a major mechanism underlying their inhibitory effect on stimulated platelets (19,56).

The results suggested that the HUVEC ADPase is a membrane-associated ectonucleotidase of the E type (57). This was verified by calcium/magnesium dependence, the ineffectiveness of specific inhibitors of P-, F-, and V-type ATPases, and the capacity to metabolize both ATP and ADP but not AMP. These findings identified the human endothelial cell enzyme as an apyrase (ATP diphosphohydrolase, ATPDase, EC3.6.1.5).

Research on ectonucleotidases had always been encumbered by difficulties in isolation related to their low abundance, relatively high activity, and sensitivity to denaturing agents (46,57-62). In 1996, Handa and Guidotti purified and cloned a soluble ATPDase (apyrase) from potato tubers (63). Sequence analysis revealed 25% amino acid identity and 48% amino acid homology with human CD39. The latter, a lymphoid cell activation antigen, had been cloned and structurally characterized by Maliszewski et al. (64). CD39 is a cell-surface glycoprotein that is expressed in activated natural killer cells, B cells, and subsets of T cells. It has also been

identified in some human endothelial cell lines (65). Based on these reports, we hypothesized that the human endothelial cell ecto-ADPase was identical to CD39.

Experimental data were developed which established identity between CD39 and the human endothelial cell ecto-ADPase (46,61,62). We tested whether antibodies against human CD39 would recognize ADPase on cultured HUVEC. Lysates from intact HUVEC were incubated with CD39 antibodies, and the resulting immunoprecipitates demonstrated ADPase activity in this assay system (46). When serial immunoprecipitation with a monoclonal antibody was used, more than 95% of the ADPase activity was removed from a purified HUVEC ADPase preparation. CD39 was responsible for more than 95% of the HUVEC ecto-ADPase activity.

Our hypothesis that the human endothelial cell ecto-ADPase was CD39 was further verified by transient transfection of COS cells with plasmids containing the human or murine CD39 sequence. Transfected COS cells acquired ADPase activity that was comparable to or greater than that of HUVEC. Microsomal membrane preparations from transfected COS cells also displayed ADPase activity.

We also compared human endothelial cell mRNA with recombinant human CD39 cDNA. Endothelial cell mRNA was analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR) using four separate primer pairs derived from the original sequence of human CD39 (64). Emphasis was placed on the NH_2 -terminal portion, which represents the enzymatic domain. Then, pHuCD39 cDNA was used for direct comparison of PCR product sizes. The PCR products of HUVEC and pHuCD39 were of similar size for each of four fragments spanning 1,144 of the 1,529 bp of the human CD39 coding sequence. Sequence analysis of gel-purified PCR products confirmed 100% identity between HUVEC CD39 and the published CD39 sequence (46).

Northern blot analyses using a probe derived from pHuCD39 cDNA revealed that mRNA for CD39 in human endothelial cells was expressed in the same band pattern as MP-1 cells. MP-1 is the Epstein-Barr virus-transformed B-cell line from which CD39 was originally cloned (64). The message from pHuCD39-transfected COS cells corresponded to the 1.9-kb cloned species.

We reasoned that if HUVEC ecto-ADPase and CD39 were identical, the CD39-bearing cells should induce blockade or reversal of platelet responsiveness to the agonist, ADP. This hypothesis was verified with the use of CD39-expressing cells, both endogenous (endothelial and MP-1) cells and COS cells transfected with pHuCD39 or its murine equivalent, pMuCD39. When any of these three types of cells was combined with platelet-rich plasma, platelet reactivity to $10 \mu\text{mol/L}$ ADP was reversed within

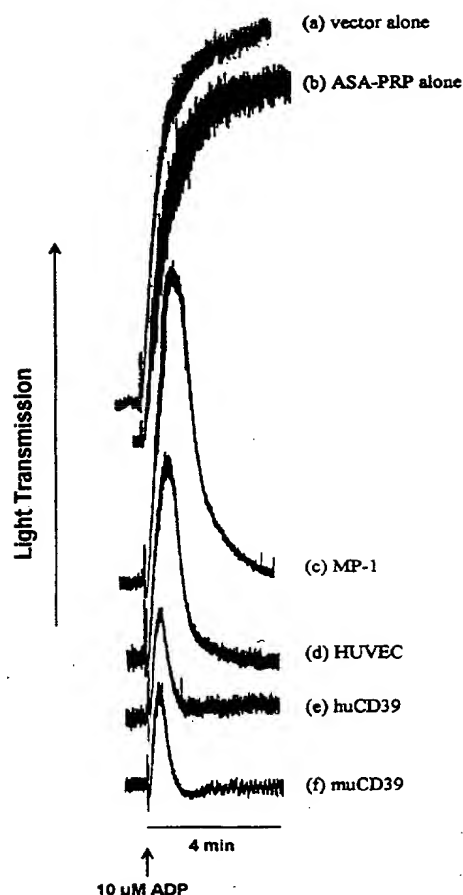


FIG. 6-8. Demonstration of blockade and reversal of platelet aggregation in response to adenosine diphosphate (ADP) by intact human umbilical vein endothelial cells (HUVEC), MP-1 cells from which CD39 was originally cloned, and COS cells transfected with full-length CD39. Aspirin-treated platelet-rich plasma (ASA-PRP) was stimulated with 10 $\mu\text{mol/L}$ ADP, and the aggregation response was measured over a 4-minute period. Addition of COS cells transfected with vector alone resulted in a full aggregation response (A), indistinguishable from the response with ASA-PRP alone (B). C: MP-1 cells reversed the aggregation response. D: Intact HUVEC also reversed the aggregation response to ADP, to a slightly greater degree than did MP-1 cells. COS cells transfected with either pHuCD39 (E) or pMuCD39 (F) demonstrated an even greater inhibitory effect on platelet responsiveness than HUVEC. This correlated with their higher degree of biochemical ecto-ADPase activity (46).

60 seconds. MP-1 cells, which express both CD39 antigen and ecto-ADPase activity, also inhibited ADP-induced platelet aggregation (Fig. 6-8).

Results of these studies are particularly pertinent for the concept highlighted in this chapter—thromboregulation. The three known thromboregulators—eicosanoids, NO, and the ecto-ADPase—have important biologic properties that merit consideration for therapeutic intervention. Aspirin treatment does eliminate the prothrombotic action of thromboxane, but it also prevents formation of PGI₂, which limits its therapeutic effectiveness. NO is an aspirin-insensitive inhibitor of platelet function, but it is inhibited itself *in vitro* and *in vivo* by hemoglobin as it rapidly diffuses into erythrocytes.

In contrast, ecto-ADPase/CD39 is aspirin insensitive and completely inhibits platelet reactivity, even when eicosanoid formation and NO production are blocked. An emerging concept in vascular biology is that ADPase/CD39 is an effective physiologic and constitutively expressed endothelial cell inhibitor of platelet reactivity. ADPase/CD39 may form the nidus for a new class of antithrombotherapeutic agents. Figure 6-9 illustrates the possible mode of action of ecto-ADPase/CD39.

Summary and Future Perspectives

This chapter has emphasized that thrombosis and atherosclerosis are essentially multicellular processes, the course of which may be modulated by varying degrees of cell contact, activation, and secretion. During the development of occlusive vascular diseases, thrombotic and proinflammatory events are biochemically linked as parts of host defense mechanisms. Functional, biochemical, and molecular biologic determinations of the interactions among cell components of thrombi and fluid-phase reactants have yielded new information relevant to the pathogenesis of thrombosis and the inflammatory response.

This information presents new therapeutic opportunities. Upregulation of endogenous ecto-ADPase/CD39 would be advantageous, as would its development as an antithrombotic agent to be administered *in vivo*. Therapeutic agents to complement aspirin administration as currently employed should prove fruitful. A "global" multicellular approach toward prevention and treatment of vascular occlusion may be conceptually more complicated than traditional unicellular modalities, but it may offer more antithrombotic potential and greater safety.

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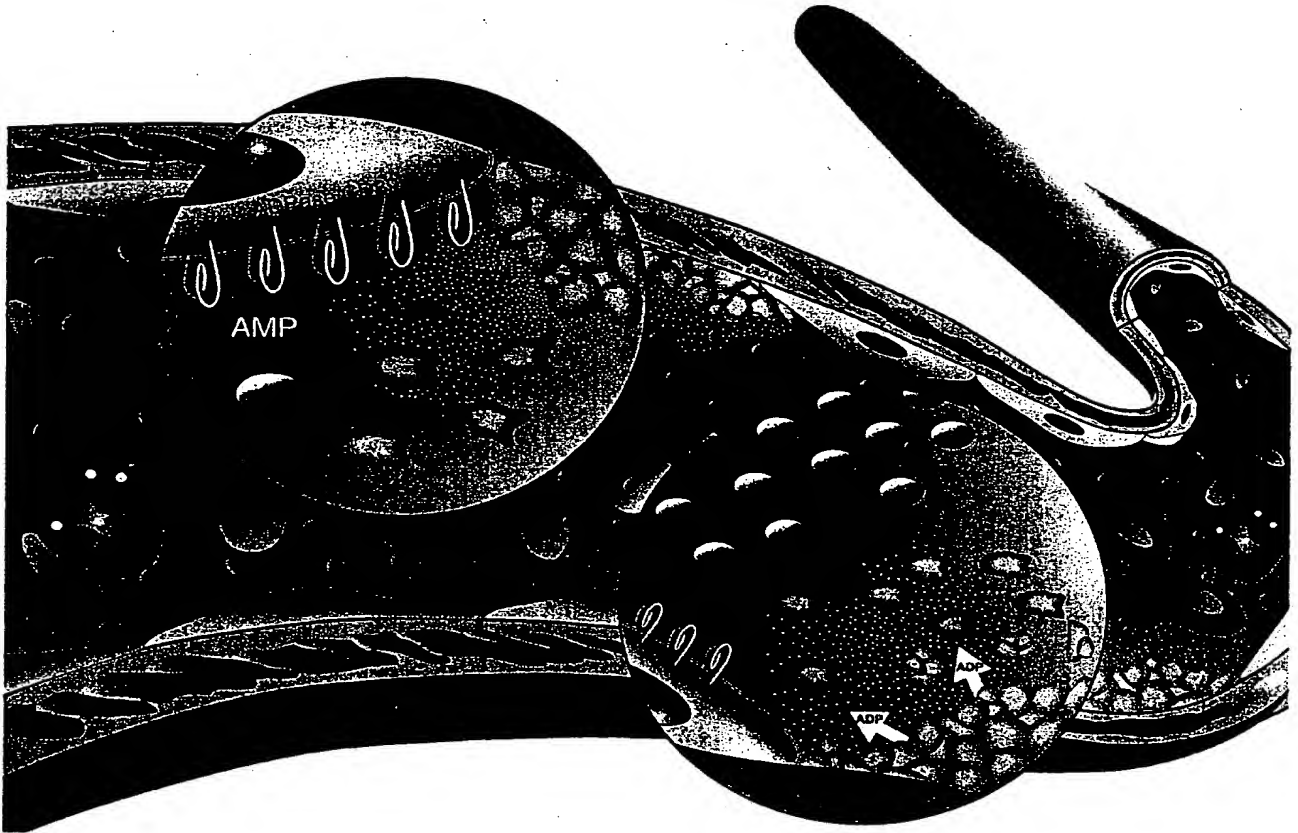


FIG. 6-9. Depiction of thromboregulation by endothelial cell ecto-ADPase/CD39. Platelet activation on or proximal to a site of vascular injury induces secretion of adenosine diphosphate (ADP) from platelet dense granules (*inset, lower right*). Released ADP activates and thereby recruits additional platelets arriving in the local microenvironment into the evolving thrombus (20). Activation and recruitment of platelets in proximity to endothelial cells is inhibited by metabolism of released ADP to adenosine monophosphate (AMP) by endothelial cell ecto-ADPase/CD39. These platelets return to an unstimulated state, thereby limiting thrombus formation (*inset, upper left*). Ecto-ADPase/CD39 has been identified and functionally characterized as a physiologic, constitutively expressed thromboregulator (46,56).

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